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Supplemental Assay Method for the Phenotypic Examination of Pseudorabies Virus for Thymidine Kinase Activity by a Plaque Selection Method

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* assay method which uses a selective media in a cell culture system to detect the presence or absence of extraneous thymidine kinase (TK)-positive pseudorabies virus (PRV) in a thymidine kinase-negative (TK-), modified-live PRV vaccine.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Water bath

2.1.2 Incubator, 36°± 2°C, high humidity, 5 ± 1% CO_2 (Model 3336, Forma Scientific Inc.)

2.1.3 Microscope, inverted light (Model CK, Olympus America Inc.)

2.1.4 Microscope, ultraviolet (UV) light (Model BH2, Olympus America Inc.)

2.1.5 Pipettor

2.1.6 Freezer, ultra-low (Revco Scientific)

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 Connective tissue, mouse, TK mutant [L-M(TK-)] cells

2.2.2 Madin-Darby bovine kidney (MDBK) cells or other susceptible cells

2.2.3 PRV Reference, Shope strain [available from the Center for Veterinary Biologics (CVB)]

2.2.4 Minimum essential medium (MEM) (Media 20030)

2.2.4.1 9.61 g MEM with Earles salts without bicarbonate

2.2.4.2 1.1 g sodium bicarbonate ($NaHCO_3$)

2.2.4.3 Dissolve with 900 ml deionized water (DI).

2.2.4.4 Add 5.0 g lactalbumin hydrolysate or edamine to 10 ml DI. Heat to 60°± 2°C until dissolved. Add to **Section 2.2.4.3** with constant mixing.

2.2.4.5 Q.S. to 1000 ml with DI; adjust pH to 6.8-6.9 with 1N hydrochloric acid (HCl).

2.2.4.6 Sterilize through a 0.22-µm filter.

2.2.4.7 Aseptically add:

1. 50 µg/ml gentamicin sulfate

2. 10 ml L-glutamine (200 mM)

2.2.4.8 Store at 2°- 7°C.

2.2.5 Growth Medium

2.2.5.1 900 ml of MEM

2.2.5.2 Aseptically add 100 ml gamma-irradiated fetal bovine serum (FBS)

2.2.5.3 Store at 2°- 7°C.

2.2.6 Hypoxanthine, aminopterin, thymine (HAT) Medium

2.2.6.1 200 ml Growth Medium

2.2.6.2 2 ml HAT media supplement (50X)

2.2.7 0.01 M Phosphate buffered saline (PBS) (Media 30054)

2.2.7.1 1.19 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)

2.2.7.2 0.22 g sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)

2.2.7.3 8.5 g sodium chloride (NaCl)

2.2.7.4 Q.S. to 1000 ml with DI.

2.2.7.5 Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH) or 1 N HCl.

2.2.7.6 Sterilize by autoclaving at 15 psi, 121°± 2°C for 35 ± 5 minutes.

2.2.7.7 Store at 2°- 7°C.

2.2.8 80% acetone

2.2.8.1 80 ml acetone

2.2.8.2 20 ml DI

2.2.8.3 Store at room temperature.

2.2.9 Tissue culture flask, 25-cm^2

2.2.10 Pipette, 10-ml

2.2.11 Graduated cylinder, 25-ml, 50-ml, 100-ml, and 250-ml, sterile

2.2.12 Swine anti-PRV fluorescein isothiocyanate-labeled conjugate (PRV Conjugate) [available from CVB]

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have experience in aseptic techniques and cell culture growth and maintenance.

3.2 Preparation of equipment/instrumentation

On the day of testing, set a water bath at 36°± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 A day prior to test initiation and a day prior to each of the 3 additional L-M(TK-) passages, seed 25-cm^2 flasks with L-M(TK-) cells, in Growth Medium, at a cell count that will produce a monolayer after 1 day of incubation. Cells used for seeding should be from monolayers that had been previously passaged every 3 to 4 days. Three L-M(TK-) control flasks and 1 L-M(TK-) flask are required for each Test Vaccine. Incubate at 36°± 2°C in a CO_2 incubator.

3.3.2 A day before the last passage, seed 25-cm^2 flasks with MDBK cells, in Growth Medium, at a cell count that will produce a monolayer after 1 day of incubation. Three MDBK control flasks and 1 MDBK flask are required for each Test Vaccine. Incubate at 36°± 2°C in a CO_2 incubator.

3.3.3 PRV Positive Control. On the day of test initiation, rapidly thaw a vial of PRV Reference in a 36°± 2°C water bath. Dilute the PRV Reference in MEM to contain 10^4 50% tissue culture infective dose ($TCID_{50}$)/100 µl.

3.3.4 Working PRV Conjugate. On the day of the fluorescent antibody (FA) Confirmatory Test, dilute the PRV Conjugate in PBS according to the Center for Veterinary Biologics (CVB) supplied Reagent Data Sheet.

3.4 Preparation of the Test Vaccine

On the day of test initiation, using a graduated cylinder, rehydrate a vial of the Test Vaccine with the supplied diluent. Incubate for 15 ± 5 minutes at room temperature.

4. Performance of the assay

4.1 First passage on L-M(TK-) cells

4.1.1 On the day of test initiation, decant Growth Media from all the L-M(TK-) flasks **except 1**, which remains unopened as a cell control. Label the unopened flask "Cell Control."

4.1.2 The initial test of a Test Vaccine will be with a single vial (a single sample from 1 vial). Inoculate 1.0 ml of the Test Vaccine into an L-M(TK-) flask labeled with the Test Vaccine identification.

4.1.3 1.0 ml of PRV Positive Control is inoculated into an L-M(TK-) flask labeled "PRV Positive Control."

4.1.4 1.0 ml of HAT Medium is inoculated into an L-M(TK-) flask labeled "Medium Control."

4.1.5 Allow inocula to absorb by incubating flasks at 36°± 2°C for 60 ± 10 minutes in a CO_2 incubator.

4.1.6 After incubation, add 9.0 ml of HAT Medium to all flasks except the Cell Control (remains unopened). Incubate all flasks at 36°± 2°C for 4 days in a CO_2 incubator.

4.1.7 During incubation, periodically observe all flasks for bacterial or fungal contamination. It is not necessary for microscopic observation of the L-M(TK-) cells. However, on examination, the L-M(TK-) cells will be rounded due to the HAT Medium. The Cell Control flask should remain normal.

4.1.8 After incubation, all flasks are frozen at -70°± 5°C for at least 2 hours. Flasks may be held at -70°± 5°C until the next passage. The Cell Control flask is discarded, as a new Cell Control flask will be used with each passage.

4.1.9 Thaw all the remaining flasks, with frequent shaking, at room temperature until completely thawed.

4.2 Repeat **Sections 4.1.1 through 4.1.9** for a total of 4 passages, inoculating each flask with 1.0 ml of the thawed cell and media suspension of the appropriate control or Test Vaccine from the previous passage instead of the initial inocula.

4.3 Passage on MDBK cells

4.3.1 From the last thawed passage (4th passage) on L-M(TK-) cells, repeat **Sections 4.1.1 through 4.1.3**, except substitute MDBK cells for the L-M(TK-) cells and inoculate each flask with 1.0 ml of the 4th passage cell and media suspension of the appropriate control or Test Vaccine instead of the initial inocula.

4.3.2 Allow inocula to absorb by incubating at 36°± 2°C for 60 ± 10 minutes in a CO_2 incubator.

Note: Do not use HAT Medium.

4.3.3 After incubation, add 9.0 ml of Growth Medium to all flasks except the Cell Control (remains unopened). Incubate at 36°± 2°C for 4 days in a CO_2 incubator.

4.3.4 Observe all flasks daily with the inverted light microscope for typical PRV CPE. The CPE of PRV is visible as grape-like clusters of rounded cells in the cell monolayer. The PRV Positive Control flask should show typical PRV CPE after 4 days. Any Test Vaccine observed to have typical PRV CPE is considered a Suspect Test Vaccine.

4.4 Confirmation of PRV CPE in Suspect MDBK Test Vaccine flasks (FA Confirmatory Test). Conduct an FA Confirmatory Test using specific PRV conjugate on any Suspect Test Vaccine. The PRV Positive Control flask, the HAT Medium flask, and the Cell Control flask are similarly examined and used as controls. **If CPE is not observed in a Test Vaccine, this procedure is not required.**

4.4.1 Decant Growth Medium from the MDBK flasks for the Suspect Test Vaccine(s), the PRV Positive Control, the HAT Medium, and the Cell Control (FA Test) flasks.

4.4.2 Rinse the FA Test flasks with PBS; decant the liquid.

4.4.3 Fill the FA Test flasks with 80% Acetone and incubate at room temperature for 15 ± 5 minutes.

4.4.4 Decant the 80% acetone from the FA Test flasks and air dry at room temperature.

4.4.5 Pipette 2 ml of working PRV Conjugate into the FA Test Flasks and incubate for 45 ± 15 minutes at 36°± 2°C in a CO_2 incubator.

4.4.6 Decant and add 20 ml of PBS to the FA Test flasks and rotate back and forth to rinse the cells.

4.4.7 Repeat **4.4.6** for a total of 5 washes.

4.4.8 Rinse the FA Test flasks in DI, decant, and allow to air dry or dry at 36°± 2°C.

4.4.9 Examine the FA Test flasks with a UV microscope.

4.4.10 A flask is considered positive for PRV if typical, nuclear, apple-green fluorescence is observed in any cell.

4.4.11 For a valid FA test, the PRV Positive Control must show typical, apple-green fluorescent infected cells.

4.4.12 For a valid FA test, the HAT Medium flask and the Cell Control flask must not show fluorescence.

4.4.13 If either **Section 4.4.11 or Section 4.4.12** criteria are not met, the test is considered a **NO TEST** and the entire test, starting with a new vial of the Test Vaccine, is repeated.

5. Interpretation of the test results

5.1 For a valid test, the HAT Medium flask and MDBK Cell Control must remain free of CPE and all flasks must remain free of bacterial or fungal contamination.

5.2 The PRV Positive Control must show CPE after passage in the MDBK cells.

5.3 For a **SATISFACTORY** test, a Test Vaccine must not show CPE after passage in MDBK cells.

5.4 Any Test Vaccine exhibiting typical PRV CPE in the initial test and found positive in a valid FA Confirmatory Test is retested in duplicate using 2 new vials of the Test Vaccine.

5.4.1 If either of the 2 retests of the Test Vaccine exhibits typical PRV CPE and is found positive in a valid FA Confirmatory Test, the Test Vaccine is **UNSATISFACTORY**.

5.4.2 If no typical PRV CPE is observed in either of the 2 retests, the Test Vaccine is **SATISFACTORY**.

5.5 Any Test Vaccine exhibiting typical PRV CPE in the initial test and found negative in a valid FA Confirmatory Test is considered **INCONCLUSIVE** for TK- activity. Repeat **Section 4.3.1** and examine for possible viral contamination.

6. Report of test results

Record all test results on the test record.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.318, U.S. Government Printing Office, Washington, DC, 2004.

7.2 Kit, S and Qavi, H. Thymidine kinase (TK) induction after infection of TK-deficient rabbit cell mutants with bovine herpesvirus type I (BHV-1): isolation of TK- BHV-1 mutants. Virol 1983; 130(2):381-389.

8. Summary of revisions

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **2.2.4.2** The amount of sodium bicarbonate ($NaHCO_3$) has been changed from 2.2 g to 1.1 g.

- **2.2.4.7** L-glutamine has been added. Penicillin and streptomycin have been removed.

- **2.2.5.2** L-glutamine has been removed.

- **2.2.11** "Syringe and needle" has been changed to "graduate cylinders"

- "Reference and Reagent Sheet" has been changed to "Reagent Data Sheet" throughout the document.

- "Test Serial" has been changed to "Test Vaccine" throughout the document.

- The refrigeration temperatures have been changed from 4° ± 2°C to 2°- 7°C. This reflects the parameters established and monitored by the Rees system.